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cDNA ENCODING P2P PROTEINS AND USE OF P2P cDNA-DERIVED ANTIBODIES AND ANTISENSE REAGENTS IN DETERMINING THE PROLIFERATIVE POTENTIAL OF NORMAL, ABNORMAL, AND CANCER CELLS IN ANIMALS AND HUMANS

Sub B'

This application is a continuation of pending provisional application 60/027,568, filed September 27, 1996.

BACKGROUND OF THE INVENTION

Differentiation in many cell lineages has been established to be a multistep process. This is perhaps best illustrated by analysis of the differentiation of 3T3T mesenchymal stem cells into adipocytes (1). Undifferentiated 3T3T cells first arrest their proliferation in the G_1 phase of the cell cycle at a distinct state prior to differentiation. Associated with this process, expression of the PPREg2 (2) lineage specific transcription factor is induced (2). Thereafter, the C/EBP family of transcription factors are expressed to induce a series of adipocyte differentiation genes that include 422, GDPH, lipoprotein lipase and adipsin (3,4,5). The resultant adipocytes are nonterminally differentiated because they can be induced to reinitiate proliferation and reenter the cell cycle. Adipocytes at the nonterminal state of differentiation can however be induced to terminally differentiate by exposure to aproliferin and thereby irreversibly lose their growth factor responsiveness (6). When terminal adipocyte differentiation occurs a marked repression in the expression of P2P proteins is evident (7). Repression in P2P protein expression has also been shown to be associated with the

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that cannot terminally differentiate, P2P expression cannot be repressed (7).

P2Ps, i.e. proliferation potential proteins, comprise a group of highly basic 35-40 kDa nuclear proteins that can bind to RNA and are associated with hnRNP particles as determined by sucrose gradient sedimentation of nuclear components (7). In this application, references to a singular P2P protein encompass the plural P2P "proteins", and vice versa. Antibodies prepared against core hnRNPs recognize P2Ps and 2D gel electrophoresis established that P2Ps are members of the A/B class of hnRNP proteins which are involved in RNA processing (7,9).

Terminal differentiation has also recently been demonstrated to require the expression of the tumor suppressor protein Rb1 (10). In studies using myoblasts derived from native animals that express Rb1 and myoblasts from transgenic animals that lack Rb1, it was established that cells lacking Rb1 cannot terminally differentiate. Instead, they are blocked at a state of nonterminal differentiation. These observations suggest that the function of Rb1 as a tumor suppressor gene product may be related to its role in the control of terminal differentiation. This possibility is supported by data showing that the Wilms' tumor suppressor gene product WT1 is also involved in the terminal differentiation of renal blastema cells during neonatal development (11). Recent reports also show that the Rb1 and WT1 proteins can be localized in the nucleus to sites of RNA processing suggesting

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that tumor suppressor mechanisms may be mediated by regulating the processing of specific mRNAs (12,13).

SUMMARY OF THE INVENTION

The invention is a novel P2P cDNA, the protein/proteins encoded by the P2P cDNA, monoclonal antibodies against P2P protein, a diagnostic method which involves detection of the DNA, an RNA transcribed by the DNA, or the protein, P2P antisense reagents derived from the P2P cDNA, and a method for gene therapy using these reagents.

The invention is partly based on evidence that the irreversible loss of proliferative potential is associated with repression in the expression of hnRNP-associated proteins that are involved in RNA processing which was published in 1989 (7). It was specifically demonstrated that the terminal differentiation of 3T3T adipocytes correlates with a markedly decreased expression of a set of proteins designated P2P, i.e. proliferation potential proteins. P2Ps were shown to have a pI of greater than 9.0, to range in size from 35 to 40 kDa and to localize to nuclear hnRNP particles as determined using sucrose gradient sedimentation methods. Additional studies established that P2Ps are recognized by the FA12 monoclonal antibody that detects purified core hnRNP proteins. The results of 2D gel electrophoresis further established that P2Ps are type A/B hnRNP proteins. P2Ps also share an epitope in common with hsp90 as determined by use of the AC88 monoclonal antibody even

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though P2Ps are not heat shock proteins. Subsequently, the terminal differentiation of human keratinocytes was also shown to be associated with a marked decrease in P2P expression (7) and P2P expression was shown to markedly decrease in association with the senescence of normal human cells (8). In contrast, it was shown that P2P expression is not repressed when malignant cells differentiate (7).

A description is provided of the cloning and characterization of the P2P cDNA. The result of this effort defined a 5173 base pair cDNA, shown in Figure 6, containing a 4214 base pair open reading frame encoding a 156.9 kDa protein. The deduced amino acid sequence of the P2P open reading frame shows a highly basic protein, i.e., pI 9.6, as predicted. Probes to the P2P cDNA detect a single 8 kb mRNA in murine kidney, liver, testes, lung and other tissues and in growing murine 3T3T mesenchymal stem cells. In contrast, P2P mRNA expression is markedly decreased when 3T3T cells undergo the terminal step in the process of adipocyte differentiation. However, P2P mRNA expression is not repressed in nonterminally differentiated adipocytes suggesting that regulation of P2P expression is associated specifically with terminal differentiation.

To establish that the P2P cDNA encodes the P2P subset of hnRNPs, a series of monoclonal antibodies was prepared to a P2P cDNA-derived fusion protein, one of which is designated C130. Other monoclonal antibodies prepared from the P2P cDNA-derived

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fusion protein are designated C50, C147, and C167. The C130 antibody was shown to detect native 35 to 40 kDa P2Ps and other higher molecular weight products of the P2P cDNA, including a low abundance ~160 kDa protein. This protein is recognized by C130 when nuclear extracts are fractionated by single stranded DNA affinity chromatography. This high molecular weight protein is thought to represent the intact product of the P2P cDNA which then appears to be processed into lower molecular weight P2Ps. Since monoclonal antibodies C130 and AC88 both detect P2Ps which are proven hnRNP's of the A/B subtype, this data provides support for the conclusion that the P2P cDNA encodes hnRNP-associated proteins. Data also show that a P2P antisense oligonucleotide selectively represses 30-40 kDa P2P expression.

Studies were next performed to determine if Rb1 might interact with P2P cDNA products. This possibility was suggested by data showing that Rb1 is involved in terminal differentiation and in other growth control mechanisms. Evidence that the P2P cDNA does encode a Rb1 binding protein was obtained by analysis of the Rb1 binding characteristic of GST-P2P fusion proteins. GST-P2P (753 to 909) was specifically shown to bind Rb1. The fact that Rb1 binding to this fusion protein is specifically competed by E1a suggest that the binding occurs to the Rb1 "pocket" domain (28,29). These data are consistent with data concerning the RBQ1 cDNA which was selected based on its ability to bind Rb1 and the fact that the

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RBQ1 cDNA shows significant homology to the 5′ portion of the P2P cDNA (23).

The deduced P2P cDNA product also contains additional interesting domains. The first of these is a cell division sequence motif [CDSM] that has been proposed to be characteristic of proteins involved in the regulation of cell division (27). Examples of proteins that contain this motif include cdc 25, *c-myc* and several viral proteins including E1a, E7 and SV40 large T antigen. The presence of the CDSM in the P2P cDNA product is consistent with the evidence showing that P2Ps are involved in regulating a cells proliferative potential. Another distinct domain encoded by the 5′ portion of the P2P cDNA is a cysteine-rich region that is related to "ring" zinc fingers (30). These zinc finger domains are thought to define protein conformation characteristics that are involved in nucleic acid binding and protein-protein interactions. These attributes are compatible with the fact the P2Ps are known to bind to single stranded DNA and to associate with other hnRNP proteins (31).

These data together suggest that the P2P cDNA can encode protein domains that are important in growth control and that can be modulated by differentiation. The fact that P2P cDNA products can bind Rb1 is highly significant especially since it has been recently shown that both the Rb1 and WT1 tumor suppressor proteins localize to sites of RNA processing as do P2Ps (12,13). Additionally, the ability of Rb1 to bind to nuclear matrix proteins

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(12,13), such as, p84 (12) and lamin A (32), is of interest since hnRNP particles are also known to be associated with the nuclear matrix (33).

It is therefore considered that the product(s) of the P2P cDNA function to bind tumor suppressors and other cell division regulatory proteins and modulate their function in regulating the processing of RNAs that effect growth control and mediate tumor suppression. Therefore, it is considered that the P2P gene product(s) would represent important regulatory factor(s) that effects many biological and pathological mechanisms including growth control, differentiation, tumor suppression and carcinogenesis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a model for the P2P cDNA and its deduced protein. The P2P cDNA consists of 5173 base pair that contains 4214 base pair open reading frame (ORF) extending from base pair 139 to 4353. Domains of the deduced 1404 amino acid protein expressed as fusion proteins are also shown. These include four GST-P2P fusion proteins and one b-galactosidase fusion protein designated C130. The amino acid residues of each fusion protein is given parenthetically.

Figure 2 shows P2P cDNA-deduced protein consisting of 1404 amino acids. A hnRNP-associated domain is encoded by amino acids 853 to 1404 and Rb1 binding domain is encoded by amino acids

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753 to 908. Potential nuclear localization signals are present between amino acids 717 to 1323 (underlined) and a cysteine-rich domain resembling a "ring" zinc finger is also present from amino acid 61 to 101 (boxed). The cell division sequence motif (CDSM) from amino acids 79 to 97 (bold) is also shown.

Figure 3 shows tissue distribution of the P2P mRNA and its specific repression by terminal adipocyte differentiation. A) A murine multiple tissue Northern blot (Clontech) was analyzed using ^{32}P-labelled random primed P2P cDNA probes under high stringency conditions. Size markers, in kilobases (kb) are shown on the left. B) total cellular RNA (20 mg) isolated from growing undifferentiated 3T3T cells (RG), quiescent serum starved undifferentiated 3T3T cells (Gs), quiescent predifferentiated 3T3T cells arrested 3T3T cells (G_0/G_D), nonterminally differentiated 3T3T adipocytes (NTD) and terminally differentiated 3T3T adipocytes (TD) were hybridized with ^{32}P-labelled random primed P2P cDNA probes under high stringency conditions. A photograph of the ethidium bromide stained gel prior to nucleic acid transfer to the nitrocellulose membrane is shown to indicate equivalent amounts of RNA in each lane.

Figure 4 shows monoclonal antibody C130 derived from a P2P cDNA fusion protein detects native P2P. Rapidly growing undifferentiated murine 3T3T total (T) cellular extracts (100 mg per lane) or nuclear (N) extracts (40 mg per lane) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes.

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Blots were probed with monoclonal antibody AC88 to detect native P2P proteins or with the P2P cDNA derived monoclonal antibody C130. The location of P2P proteins and heat shock protein 90 (HSP90) are indicated. Size standards are shown in kilodaltons (kd).

Figure 5 shows GST-P2P (753-909) specifically binds Rb1 through the "pocket domain". A) Aliquots of a lysate prepared from K562 cells (1 x 10^7 cells/sample) were incubated with the glutathione S-transferase leader sequence (GST), or with GST-P2P (1-332), GST-P2P (494-688), GST-P2P (918-1095) or GST-P2P (753-909). As precipitation controls, aliquots of the K562 lysate were immunoprecipitated with anti-Rb1 antibody IF8 or normal rabbit sera. Bound proteins were separated by electrophoresis in a 7% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. An aliquot of the K-562 crude lysate was included as a positive control for Western analysis. Proteins were visualized by probing the blot with anti-Rb1 antibodies IF-8 or C15. B) Binding of GST-P2P (753-909) to Rb1 is completed by E1a protein. Aliquots of a K562 lysate were incubated as above with GST or GST-P2P (752-909) in the presence (+E1a) or absence (-E1a) of purified adenovirus E1a protein. Bound proteins were separated by electrophoresis in a 7% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Proteins were visualized by probing the blot with anti-Rb1 antibodies IF8 or C15.

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Figure 6 shows the nucleotide sequence of the P2P cDNA. The nucleotide sequence contains an open reading frame and additional 3' and 5' untranslated regions of the P2P cDNA.

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

Cell Lines and Cell Culture Methods

The Balb/c 3T3T mesenchymal stem cell line has been previously described in detail (14). Growing monolayer cultures of these cells are maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DME, Sigma Chemical, St. Louis, MO) supplemented with 10% bovine calf serum (BCS, Hyclone, Logan, UT). To prepare quiescent undifferentiated 3T3T cells they were cultured in DME containing 0.5% BCS for 3 to 4 days at low cell densities of 1 x 10^4 cells/cm^2. In some studies, growing undifferentiated cells were treated with 50 mg/ml of P2P antisense or sense oligonucleotides.

The human hematopoietic stem cell line K-562 has also been well characterized (15) and these cells are grown as suspension cultures in RPMI 1640 supplemented with 10% BCS.

Cell Differentiation

The procedures to induce 3T3T cells to undergo differentiation into adipocytes has previously been described (16). This process involves three steps: (a) predifferentiation growth arrest, (b) nonterminal differentiation and (c) terminal

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differentiation. These steps occur in a parasynchronous manner during a 3-10 day interval after low density, growing cells are cultured in heparinized DME containing 25% human plasma (HP) on ethylene oxide treated petri dishes (16). It is possible to obtain highly enriched populations of cells at each of the differentiation states described above by using well documented modifications of these culture conditions and reagents (1,14,16). These methods were used to prepare cell populations for the current studies.

Preparation of Cellular Lysates

Cellular lysates were prepared as described by Kaelin et al. (17). Growing murine Balb/c 3T3T, and human K562 cells were washed twice with ice-cold PBS, and lysed for 30 minutes at 4° C in ice-cold EBC buffer (50 mM Tris [PH 8.0], 120 mM NaCl, 0.5% NP-40, 200 mM sodium orthovanadate) containing 10 mg/ml of the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (Sigma). The lysates were cleared of nuclei and debris by centrifugation at 14,000 x g for 15 minutes at 4° C.

P2P cDNA Cloning and Sequencing

To clone P2P related sequences, approximately 1 x 10^6 plaques from an oligo(dT) random primed lgt11 murine 3T3 fibroblast cDNA expression library (Clontech, Palo Alto, CA) were screened using standard procedures (18) with monoclonal antibody AC88 or FA12. The AC88 antibody, generated against hsp90, cross-reacts

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with the P2P proteins and has previously been described (19). FA12 also recognizes P2Ps and was prepared against core hnRNP proteins (20).

Briefly, *E. coli* strain Y1090 was infected with recombinant λgt11 phage, plated on LB plates and incubated at 42° C for 3 hours. Subsequently, the plates were overlaid with nitrocellulose filters saturated in 10 mM isopropyl b-D-thiogalactoside (IPTG) and incubated an additional 3 hours at 37° C to induce the expression of the b-galactosidase fusion proteins. At the end of this period the nitrocellulose filters were air dried for 1 hour at room temperature and subsequently incubated in blocking buffer for 1 hour. Antibody probing was performed at room temperature in the blocking buffer for 2 hours. The filters were washed three times in blocking buffer and probed with an alkaline phosphatase conjugated rabbit anti-mouse IgG (for AC88) or rabbit anti-mouse IgM (for FA12) and the filters developed as previously described (18). AC88 positive clones were further screened with monoclonal antibody FA12. Clones positive for both AC88 and FA12 were identified and isolated by multiple rounds of plaque purification.

The resulting P2P cDNAs were subcloned into the EcoRI site of the pGEM3 vector (Promega, Madison, WI) and restriction endonuclease sites were mapped. Various restriction endonuclease fragments of the P2P cDNAs were also subcloned into the vectors

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Additional 5' P2P sequences were cloned using the Rapid Amplification of cDNA Ends (5' RACE) method (22). For RACE, gene-specific oligonucleotides were used to prime first strand cDNA synthesis from murine 3T3T total RNA using the cDNA Cycle kit (Invitrogen, San Diego, CA) and 5'-RACE was performed using a variety of different primer sets. Amplified products were characterized by size analysis, cloned into the pCRII vector (Invitrogen) and their DNA sequences were determined. Throughout this sequencing procedure periodic searches of the DNA databases using the BLAST programs were performed for related sequences. As the sequencing of the 5' end of the P2P cDNA was being completed, one significant homology was discovered. A human cDNA, designated RBQ1 (23), was found to have extensive homology to a 5' region of the murine P2P cDNA. Therefore, primers for the 5' most region of RBQ-1 were also used in characterizing the P2P cDNA using RT-PCR.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated from growing cells, quiescent undifferentiated cells, cells at the nonterminal differentiation state, and terminally differentiated cells. Total cellular RNA (20 mg) from each sample was denatured and fractionated on 1.2% formaldehyde-agarose gels and transferred by blotting to nitrocellulose filters. Blots were prehybridized for

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at least two hours at 42^0 C with 5X Denhardts solution, 5X SSC, 50% formamide, 25 mM potassium phosphate, and 100 mg/ml denatured salmon sperm DNA. Hybridizations were carried out overnight at 42^0 C in the same solution containing 10% dextran sulfate and random-primed ^{32}P-labeled P2P cDNA probes. After hybridization the filters were washed and autoradiographed with intensifying screens at -70^0 C. Tissue specific expression of the P2P mRNA was determined using a mouse multiple tissue Northern blot (Clontech) according to the manufacturer's protocol.

Fusion Protein Expression for Monoclonal Antibody Production

P2P cDNAs were removed from the pGEM vectors using restriction endonuclease Eco RI and ligated downstream from the bacteriophage T7 gene 10 promoter and translation initiation site in the Eco RI site of the pET5a, b and c vectors using standard procedures. Bacterial clones were screened for the presence and orientation of the inserts by digestion with EcoRI and PstI. Individual clones containing the cDNAs in all six possible reading frames were used for subsequent analysis. Expression was achieved using the procedure described by Studier et al (24). For each of the cDNAs only one reading frame, which corresponded to the largest open reading frame, resulted in expression of a fusion protein antigenically related to P^2Ps. These fusion proteins were then used to produce an antiP2P specific monoclonal antibody, C130, at the University of Tennessee Memphis Molecular Resource Center

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Hybridoma Laboratory. Additional monoclonal antibodies, such as those designated C50, C147, and C164 were produced by injection of the transcript product of the P2P cDNA into mice, in the same method as for production of the C130 antibody. Polyclonal antibodies which bind to the P2P transcript are also conceived. The bacterial expression system consisting of the pET5 series of expression vectors, the bacteriophage CE6 and E. coli strain HMS174 were gifts from Dr. F. W. Studier.

Expression of P2P-GST and 6X-His-E1a Fusion Proteins

P2P cDNA sequences coding for P2P peptides were generated using RT-PCR and ligated into the pGEX-KG vector to generate the following GST fusion proteins of specific cDNA sequences given parenthetically: GST-P2P(1-332), GST-P2P(494-688), GST-P2P(753-909) and GST-P2P(918-1095). Expression and purification of the GST-fusion proteins was performed as described (25). Fresh overnight cultures of E. coli BL21 transformed with either pGEX-KG or one of the above mentioned pGEX-P2P recombinants were diluted 1:10 in LB medium containing 100mg/ml ampicillin and incubated at 30^0 C with shaking for one hour. Fusion protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM and the cultures grown for an additional 3 hours (17). To recover the fusion proteins, the bacterial cultures were sedimented by centrifugation at 5000 x g for 5 minutes at 4^0 C and resuspended in 1/10 volume of NETN buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM

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EDTA, 0.5% NP-40). The cells were lysed on ice by mild sonication and cellular debris was removed by centrifugation at 10,000 x g for 5 minutes at 4^0 C. Glutathione-agarose beads were washed three times and resuspended (1:1 [V/V]) in NETN; bacterial supernatants were then mixed with the glutathione-agarose beads and rocked at 4^0 C for one hour to allow the fusion proteins to bind. The beads were finally washed five times with NETN buffer. For analysis of bound bacterial GST- or 6X-His proteins, the beads were boiled in 1x SDS sample buffer, analyzed by SDS polyacrylamide gel electrophoresis and then the proteins were visualized by staining with Coomassie blue.

An E1a vector used to express the E1a protein as a 6X-His fusion protein was the gift of Dr. Margaret Quilan, University of Tennessee, Memphis. Expression and purification of the fusion protein was carried out using the His-Bind Kit following the manufacturer's protocol (Novagen, Madison, WI).

pRb1-Binding Assay and Immunoprecipitation

Glutathione S-transferase (GST)-P2P fusion proteins were expressed and recovered on glutathione-sepharose beads as described above. Whole-cell lysates of K562 cells ($1x10^7$ cells/sample) were rocked with the beads for 1 hour at 4^0 C and then washed five times with NETN buffer. The beads were then boiled in 1X SDS loading buffer and the proteins separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Competition experiments

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were performed by adding an excess of the 6X-His E1a fusion protein to the cellular lysates prior to the addition of the GST-P2P fusion proteins. The Rb1 protein was visualized by immunoblotting using anti-Rb1 antibodies IF8 or C15 (Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies were also used to immunoprecipitate native Rb1 from the cellular lysates to serve as a positive control following the manufacturers' protocol.

Results

Cloning and Characterization of the P2P cDNA

Figures 1 and 2 provide a summary of the characteristics of the P2P cDNA that has been cloned. To clone the P2P cDNA, a 3T3 cDNA lgt11 library (Clontech) was screened using the AC88 monoclonal antibody that detects both P2Ps and hsp90 (7,19). AC88 positive clones were rescreened with the monoclonal antibody FA12 against core hnRNP proteins, which was previously shown to also react with the P2Ps (7,20). Two independent clones, designated clone A (1398 bp) and clone B (1943 bp), were found to be recognized by both antibodies. Nucleotide sequencing of the cDNAs showed that the 3' most region of clone A and the 5' most region of clone B were 100% homologous over a 863 base pair region, suggesting that these were overlapping clones derived from a single RNA species. The overlapping clones were joined through a unique HindIII restriction endonuclease site in the overlapping region to

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generate 2478 base pair cDNA clone. This includes a 1658 base pair open reading frame and 820 base pair of 3' untranslated sequence.

Additional screens of the cDNA library using this cDNA as the probe failed to give new clones with any additional 5' cDNA sequence. Therefore, the cDNA clone was extended towards the 5' end using RACE (Rapid Amplification of cDNA Ends) methods whereby gene-specific oligonucleotides were used to prime first strand cDNA synthesis from murine 3T3T total RNA and 5'-RACE was performed. Amplified products were cloned and their DNA sequences were determined. This extended the 5' sequence by 1015 base pairs and a GST-P2P fusion protein derived from this region was found to bind Rb1, i.e., GST-P2P (753-909) [Figure 1].

Throughout the sequencing procedure, periodic searches of the DNA databases using BLAST programs were also performed to search for related sequences especially those encoding Rb1 binding domains. One significant homology was found with a human cDNA, designated RBQ1 (23), which was isolated by its Rb1 binding characteristics. Primers to the 5' end of RBQ1 were therefore used to further extend the P2P cDNA sequence using RT-PCR methods to give a 5173 base pair P2P cDNA.

Analysis of this cDNA reveals a single long open reading frame extending from an ATG codon at base 139 to a termination codon at base 4353. The presence of two in-frame stop codons near the 5' end of the cDNA and several in-frame stop codons at the 3' end of the cDNA suggest that the cDNA contains the entire coding

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region of the gene. This open reading frame has the potential to code for 1404 amino acids to generate a protein having a predicted molecular mass of 156.9 kDa. The deduced amino acid sequence of the protein is shown in Figure 2. This highly basic protein (pI, 9.6) has multiple potential nuclear localization signals between amino acids 717 and 1323 which is in agreement with previous findings that P2Ps represent a subset of nuclear hnRNP proteins (7). In addition, computer analysis of the sequence of the P2P cDNA-derived open reading frame shows a unique cysteine-rich domain near the amino terminus (amino acids 61 to 101) which closely resembles the consensus sequence of the "ring" class of Zn^{++} finger domains (26) and another domain near the amino terminus (amino acids 79 to 97) that has been implicated in cell growth control, i.e., the cell division sequence motif [CDSM] (27).

P2P mRNA Expression in Multiple Tissues and Repression by Terminal Adipocyte Differentiation

To establish the tissue distribution and the size of the P2P mRNA, a mouse multiple tissue Northern blot was probed with a P2P cDNA probe. A single 8kb mRNA was found in all tissues examined. Very low, but detectable, levels of P2P mRNA were found in kidney, brain, and spleen while moderate levels of P2P mRNA were found in heart, lung, liver and skeletal muscle. The highest levels of P2P mRNA expression were detected in testis (Figure 3A).

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The use of probes to different 3' and 5' P2P cDNA domains detected the same 8 kb RNA by Northern blotting (data not shown).

To determine if terminal adipocyte differentiation has an effect on P2P mRNA expression, total RNA was isolated from rapidly growing 3T3T cells, quiescent serum-starved undifferentiated 3T3T cells, quiescent predifferentiated 3T3T cells, nonterminally differentiated 3T3T adipocytes, and terminally differentiated 3T3T adipocytes. Then Northern analysis was used to compare P2P mRNA levels in cells at these states. Figure 3B shows that the 8 kb P2P mRNA is expressed in all specimens except those derived from cells at the terminal stage of adipocyte differentiation where its expression is markedly repressed. This result is in agreement with previous findings that P2P protein expression is repressed when murine 3T3T mesenchymal stem cells and normal human keratinocytes irreversibly lose their proliferative potential in association with terminal differentiation (7) or senescence (8).

A Monoclonal Antibody Produced Against A P2P cDNA-derived Fusion Protein Reacts With Native P2Ps.

The carboxy-terminal portion of the P2P cDNA ORF (base pairs 2695 to 4353) were subcloned into the pET5 series expression vector. In this system, the cDNA was placed proximal to the bacteriophage T7 gene 10 translation initiation site such that individual plasmids were isolated containing the cDNA in all six reading frames in phase with the gene 10 protein product.

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Expression of the protein encoded in each reading frame was obtained by infecting E. Coli strain HMS174 harboring the recombinant plasmid with the bacteriophage CE6 as described above. This bacteriophage is a lambda-derived phage containing the gene for T7 RNA polymerase. Infected bacteria containing the recombinant pET5 vectors produce the T7 RNA polymerase which in turn directs the expression of fusion proteins between the T7 gene 10 protein and the reading frame of the cDNAs. Only one reading frame which corresponds to the 3' end of the large open reading frame [Figure 1], resulted in expression of fusion proteins antigenically related to P2Ps. The fusion protein was electroeluted from preparative gels and used to produce a P2P-specific monoclonal antibody at the University of Tennessee, Memphis, Molecular Resource Center Hybridoma Laboratory.

One hybridoma so generated was reactive against the purified fusion protein. The antibody, termed C130, was therefore used to probe 3T3T nuclear and total cell extracts by Western analysis. Figure 4 shows that the C130 monoclonal antibody specifically detects native P2P proteins in a manner similar to the pattern seen with AC88. However, C130 and AC88 recognize separate epitopes because C130 detects only P2Ps whereas AC88 shows cross-reactivity to heat shock protein 90. These data support the conclusion that the cloned P2P cDNA encode hnRNP-related P2P peptides.

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Evidence that the P2P cDNA Encodes a Rb1 Binding Peptide Using A P2P-GST Fusion Protein

Because Rb1 is required for muscle cell terminal differentiation (10) and data showing that P2P expression is modulated during terminal adipocyte differentiation state, studies were performed to determine if P2P cDNA products might interact with Rb1. To accomplish this GST-P2P fusion proteins were periodically produced to different P2P cDNA domains. Cellular lysates were prepared from human K-562 hematopoietic stem cells which contain abundant Rb1 protein and these lysates were then precipitated with each of the four GST-P2P fusion proteins, i.e. GST-P2P (1-332), (484-688), (753-908) and (918-1095) as illustrated in Figure 1. The lysates were also precipitated with GST protein alone as a negative control in these experiments. Figure 5A demonstrates that one fusion protein, GST-P2P (753-909), specifically precipitates a protein that is detected by the anti-Rb1 antibody IF8. Figure 5a also shows that the GST-P2P (753-909) fusion protein preferentially binds the hypophosphorylated form of Rb-1 which is primarily expressed in the G_1 phase of the cell cycle thus suggesting a possible physiological role for the interaction of P2P cDNA products and Rb1 in the control of cell growth.

Most proteins that associate with the hypophosphorylated form of Rb1 bind to a region of Rb1 that has been termed the "pocket" domain (28). To determine if the interaction between Rb1

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and GST-P2P (753-909) occurs through the Rb1 "pocket" domain, competition experiments were conducted using purified viral E1a protein. E1a is known to bind specifically to the Rb1 pocket domain and to inhibit cellular proteins from binding to this region (29). Figure 5B shows that the interaction between the GST-P2P(753-909) fusion protein and Rb1 is blocked by the addition of purified E1a protein. This inhibition is specific for the E1a protein since the addition of another protein, dihydrofolate reductase, did not block the interaction of Rb1 and the GST-P2P fusion protein (data not shown). Therefore, GST-P2P(753-909) binds specifically to the hypophosphorylated form of Rb1 and this interaction occurs through the Rb1 "pocket" domain.

Gene Therapy Using P2P cDNA-Derived Antisense Oligonucleotide Reagents

P2P mRNA and P2P protein is expressed in cells that have proliferative potential regardless of whether they are in a growing or quiescent state. Conversely, the expression of P2P cDNA products is repressed in cells that have lost their proliferative potential as a result of terminal differentiation or senescence. In contrast, transformed cells with malignant characteristics, especially SV40 transformed cells that lack the ability to terminally differentiate or senesce, lack the ability to repress P2P expression. It is conceived, therefore, that the proliferative potential of cancer cells, in general, may be blocked if P2P

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expression is repressed by the use of antisense oligonucleotide reagents that are targeted to bind to specific domains of the P2P mRNA to block its translation.

The P2P antisense oligonucleotide [5' CAGCAGGAGCTGTGTT ' 3 cDNA (3424-3409)] and a P2P sense oligonucleotide [5' CTACTAAGCCATCGGC ' 3 (3560-3575)] have been prepared, isolated, and studied, as shown below in Table I. The antisense oligonucleotides are prepared by Jude Labs (Memphis, TN) and BioSynthesis (Louisville, TX). These oligonucleotides (15-50 mg/ml) were added to the culture media of growing 3T3T cells for various times up to 9 days and the effect of these treatments on P2P expression was determined by Western blotting using the AC88 antibody to detect P2Ps.

Table 1

Selective Repression of P2P Expression
With Antisense Oligonucleotides

	Repression of P2P Expression	Repression of Control Protein hsp90
P2P Antisense	83%	0%
P2P Sense	6%	0%

Additional data also suggests that a P2P antisense oligonucleotide can repress cellular proliferation by greater than 50%, whereas a P2P sense oligonucleotide has no effect. Thus, P2P

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antisense reagents which bind to a domain of the open reading frame of P2P cDNA can be used to repress P2P expression and cellular proliferation, which indicates that the repression of P2P expression may be able to repress the proliferative potential of normal, nontransformed cells, abnormal cells, and cancerous cells both in vitro and in vivo. The results of these studies establish the therapeutic value of P2P antisense reagents for the treatment of proliferative diseases, including cancer.

The ATCC Accession Number for monoclonal antibody C130 is:

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